FGF-dependent midline-derived progenitor cells in hypothalamic infundibular development

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FGF-dependent midline-derived progenitor cells in hypothalamic infundibular development

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SUMMARY

The infundibulum links the nervous and endocrine systems, serving as a crucial integrating centre for body homeostasis. Here we describe that the chick infundibulum derives from two subsets of anterior ventral midline cells. One set remains at the ventral midline and forms the posterior-ventral infundibulum. A second set migrates laterally, forming a collar around the midline. We show that collar cells are composed of *Fgf3*$^+$ SOX3$^+$ proliferating progenitors, the induction of which is SHH dependent, but the maintenance of which requires FGF signalling. Collar cells proliferate late into embryogenesis, can generate neurospheres that passage extensively, and differentiate to distinct fates, including hypothalamic neuronal fates and *Fgf10*$^+$ anterior-dorsal infundibular cells. Together, our study shows that a subset of anterior floor plate-like cells gives rise to *Fgf3*$^+$ SOX3$^+$ progenitor cells, demonstrates a dual origin of infundibular cells and reveals a crucial role for FGF signalling in governing extended infundibular growth.

KEY WORDS: FGF, Floor plate, Hypothalamus, Chick

INTRODUCTION

In the adult, homeostasis, i.e. the control of the body's internal environment, is mediated through the hypothalamo-pituitary neuraxis. A central feature of this axis is the projection of hypothalamic axons through an evagination of the ventral hypothalamic floor termed the infundibulum (Pelletier, 1991). Despite its key role in body function, little is understood of the cellular and molecular events that orchestrate formation of the infundibulum and that sculpt and maintain it over time.

Much of the ventral hypothalamus arises from anterior ventral midline floor plate-like cells (Manning et al., 2006). As one of the major organisers of the CNS, floor plate (fp) cells instruct neural cells to acquire distinctive fates and establish the elaborate neuronal networks that underlie CNS function (Jessell and Dodd, 1990; Ericson et al., 1997; Colamarino and Tessier-Lavigne, 1995; Placzek and Briscoe, 2005). Numerous lines of evidence suggest that the fp is a non-uniform population, with cells along the anterior-posterior axis displaying distinct characteristics (for a review, see Placzek and Briscoe, 2005). In the forebrain, fp cells are particularly heterogeneous and show dynamic changes in their molecular profiles and behaviour (Kapsimali et al., 2004; Manning et al., 2006; Ohyama et al., 2008). This raises the possibility that subsets of anterior fp contribute to defined ventral forebrain structures, including the infundibulum.

In all vertebrates examined, FGFs are expressed within both fp-derived cells of the ventral hypothalamic midline (Manning et al., 2006) and the forming infundibulum (Tannahill et al., 1992; Ohuchi et al., 2000; Herzog et al., 2004; Theil et al., 2008; Tsai et al., 2011), where they may play multiple roles in the neuroendocrine hypothalamus. FGFs act as spatial and proliferative cues for progenitors within Rathke's pouch, which is the precursor of the anterior pituitary (Ericson et al., 1998; Norlin et al., 2000; Zhu et al., 2007), and additionally promote diverse aspects of specification of neuroendocrine neurons (Tsai et al., 2011). Similarly, emerging evidence supports a function for FGF signalling in development of the infundibulum itself. In zebrafish, FGF signalling is required for the expression of Lhx2 (Seth et al., 2006), a Lim homeodomain transcription factor that is expressed widely in the ventral hypothalamus and that in mouse is required for appropriate proliferation and formation of the infundibulum. Mouse mutants that lack LHX2 expression show unusually high levels of proliferation in the ventral diencephalic floor, with concomitant failure of infundibular evagination (Zhao et al., 2010). In *Fgf10* knockout mice, furthermore, the infundibulum similarly fails to evaginate fully and infundibular cells undergo apoptosis (Ohuchi et al., 2000). These studies suggest a link between local proliferation and sculpted formation of the infundibulum, but an integrated cellular mechanism linking these events remains unclear: LHX2 and *Fgf10* are broadly expressed within the infundibulum and ventral diencephalon and could exert their actions on a number of cell types.

Elsewhere in the CNS, and particularly well-described in the posterior neural tube, FGFs play a key role in neural proliferation (Mathis et al., 2001; Diez del Corral et al., 2003; Akai et al., 2005; Cayuso and Marti, 2005), where they govern expression of SOX genes, HMG-box transcription factors that affect neural proliferation and the maintenance of neural stem/progenitor cell renewal (Bylund et al., 2003; Graham et al., 2003; Ellis et al., 2004; Pevny and Placzek 2005; Scott et al., 2010). In the anterior neural tube, the SoxB1 transcription factor, SOX3, is expressed in the hypothalamus, around the forming infundibulum, and has been previously implicated in infundibular formation. In mouse *Sox3* conditional knockouts, the infundibulum and adjacent ventral hypothalamus are thinner and show reduced proliferation rates

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(Rizzoti et al., 2004). This raises the possibility that SOX3 might maintain normal proliferation rates in ventral hypothalamic precursors and hence govern infundibular morphogenesis. Human studies support this idea, showing that aberrant dosage of SOX3 leads to infundibular hypoplasia and abnormal posterior pituitary development (Woods et al., 2005; di Iorgi et al., 2009). As yet, no study has analysed whether FGF signalling and SOX3 expression are integrated in governing proliferation around the infundibulum.

Here we analyse formation of the infundibulum in the embryonic chick. We describe a dual origin of infundibular cells from two populations of ventral midline floor plate-like cells that differ markedly in behaviour. One population persists at the ventral midline and forms the posterior-ventral (p/v) infundibulum. A second population gives rise to a collar of cells that express *Fgf3* and SOX3 and are capable of extensive proliferation, with some collar cell descendants differentiating to *Fgf10*$^{+}$ anterior-dorsal (a/d) infundibular cells over an extended period, and other collar cells being retained at the neck of the infundibulum. SOX3^{+} collar progenitors require FGF signalling for their maintenance and proliferation. Together, our studies reveal a mechanism to explain the growth and functional characteristics of the infundibulum.

MATERIALS AND METHODS

Fate mapping

Hamburger and Hamilton stage (st) 9 embryos were fate mapped with DiI/DiO (Molecular Probes) as described (Manning et al., 2006) and allowed to develop up to embryonic day (E) 7 (n=40 embryos; 5-7 per time point). In neurosphere assays, DiI was targeted to st 9 prosencephalic neck cells, embryos incubated until E4, and collar cells dissected for neurosphere generation (n=10).

Transplantation experiments

Hypothalami were isolated with Dispase (Roche; 1 mg/ml, 40 minutes) from Roslin Green chicken embryos and collar or prospective p/v infundibular cells subdissected and transplanted into the collar of isolated wild-type (wt) hypothalami (n=14 and n=5 for homochronic and heterochronic grafts, respectively). Tissue was cultured for 72 hours in Matrigel (BD Biosciences). In situ hybridisation analysis for *Fgf10* was carried out prior to detection of GFP with anti-GFP antibody (Novacastra; 1:200).

Explant cultures

Explants of fp, collar or prospective p/v infundibulum were isolated from surrounding tissues (including Rathke's pouch; $n\geq6$) after Dispase treatment and cultured in collagen (Placzek et al., 1993). SU54502 (Calbiochem; 10 or 20 μM in DMSO), FGF10 and FGF3 (R&D Systems; 10 and 100 ng/ml, respectively), FGF10- or FGF3-blocking antibodies [Santa Cruz Biotechnology; 50 ng/ml (Harada et al., 2002)] were added to the culture medium.

Cell proliferation assays

Explants were cultured for 16 hours and pulsed with 10 μM BrdU for 1 hour prior to fixation.

Immunohistochemistry and in situ hybridisation

Embryos, explants and neurospheres were analysed by immunohistochemistry and in situ hybridisation according to standard techniques (Placzek et al., 1993; Manning et al., 2006). Following cryostat sectioning (15-20 μm), the following antibodies were used: anti-SHH (1:50; 5E1, DSHB); anti-NKX2.1 (1:2000) (Ohyama et al., 2005); anti-PAX6 (1:50; DSHB); anti-PH3 (1:1000; Upstate); anti-BrdU (1:200; Novacastra); anti-TBX2 (1:1000; C. Goding) (Manning et al., 2006); anti-Musashi (1:200; Abcam); anti-SOX3 (1:500; T. Edlund, Umea, Sweden; Abcam); anti-TUJ1 (1:1000; Calbiochem); anti-pMAPK (1:50; Cell Signaling); anti-SOX2 (1:500; Chemicon); anti-GFP (1:200; Novacastra); anti-P27 (1:1000; BD Biosciences); and anti-cCaspase 3 (1:1000; Cell Signaling). Secondary antibodies (1:200; Jackson ImmunoResearch) were conjugated to Cy3 or FITC. Images were taken using a Zeiss ApoTome or Olympus BX60 and Spot RT software v3.2 (Diagnostic Instruments). For 3D reconstruction, overlap, channel visibility and orientation were performed using Image-Pro Analyser and Makromedia Fireworks; 3D rendering was performed using Volocity (version 5.4.1, Perkin Elmer). Following development, embryos or neurospheres were analysed as wholemount preparations or cryostat sections.

Neurosphere cultures

Tissues were dissected as for explants, trypsinised and mechanically dissociated. Cells were filtered, plated at 90,000-210,000 cells/well into ultralow-binding plates (Nunc) in DMEM/F12 medium supplemented with B27/N21 and with 1:100 chick embryo extract, 20 ng/ml bFGF (FGF2) and 20 ng/ml EGF (Molofsky et al., 2003) and incubated for 7 days at 37°C, 5% CO_2. Neurospheres were passaged after mechanical dissociation. For differentiation, spheres were plated onto poly-D-lysine and fibronectin in medium without EGF and cultured for 7 days.

Scanning electron microscopy (SEM)

Hypothalami were fixed in 3% glutaraldehyde for 4 hours at 4°C, dehydrated, dried, mounted and coated with 25 nm gold (Edwards S150B sputter coater). Specimens were imaged with a Philips XL-20 scanning electron microscope at an accelerating voltage of 20 kV.

RESULTS

Dual origin of infundibular cells from anterior floor plate

To address whether anterior fp cells contribute to the infundibulum, we performed fate-mapping studies in chick embryos, tracing cells from st 9 until E7 (Fig. 1A-C), when the infundibulum is structurally distinct (Fig. 1D). DiI-labelled cells were detected throughout the infundibulum, with highest levels in posterior-ventral (p/v) regions (Fig. 1C).

SEM revealed that the infundibulum first becomes apparent at E4.5 (Fig. 1Di,ii) and that at E7 two morphologically discrete infundibular cells can be detected: elongated cells that extend along and immediately adjacent to the midline in the p/v infundibulum, and rounded cells in the a/d infundibulum (Fig. 1Diii,iv). To investigate the origin of these cells, we refined the fate mapping, targeting small populations of anterior fp at st 9 with DiI/DiO and analysing embryos after increasing time periods (Fig. 1E-P). These analyses revealed a differential behaviour of fp cells that are initially apposed. Fp cells just posterior to the prosencephalic neck (green cells, Fig. 1E,F) remained at the ventral midline (Fig. 1F inset, Fig. 1G-M) and by E7 contributed exclusively to midline cells of the p/v infundibulum (Fig. 1N-P). By contrast, descendants of fp cells that were at the prosencephalic neck at st 9 (red cells, Fig. 1E,F) moved laterally/posteriorly to form a semi-ellipse of cells. These were initially apposed to the ventral midline population (Fig. 1F inset, Fig.1G) but appeared to become progressively dispersed from it with time (Fig 1H).'

To analyse this behaviour in detail, we examined serial sections and performed 3D reconstructions over the period E3.5-4.5. This revealed that, from anterior to posterior, and over time, clusters of DiI-labelled cells become increasingly displaced from the ventral midline by their descendants (Fig. 1I-M and see Movie 1 in the supplementary material). Tracing to E7 revealed that cells from the prosencephalic neck give rise either to clusters of strongly labelled cells at the top of, or immediately adjacent to, the a/d infundibulum (Fig. 1N,O, arrows) or to scattered descendants in the a/d infundibulum and adjacent hypothalamus (Fig. 1N, right-hand panel). We term the region occupied by these strongly labelled cells the 'collar zone' (white arrows in schematic, Fig. 1P).

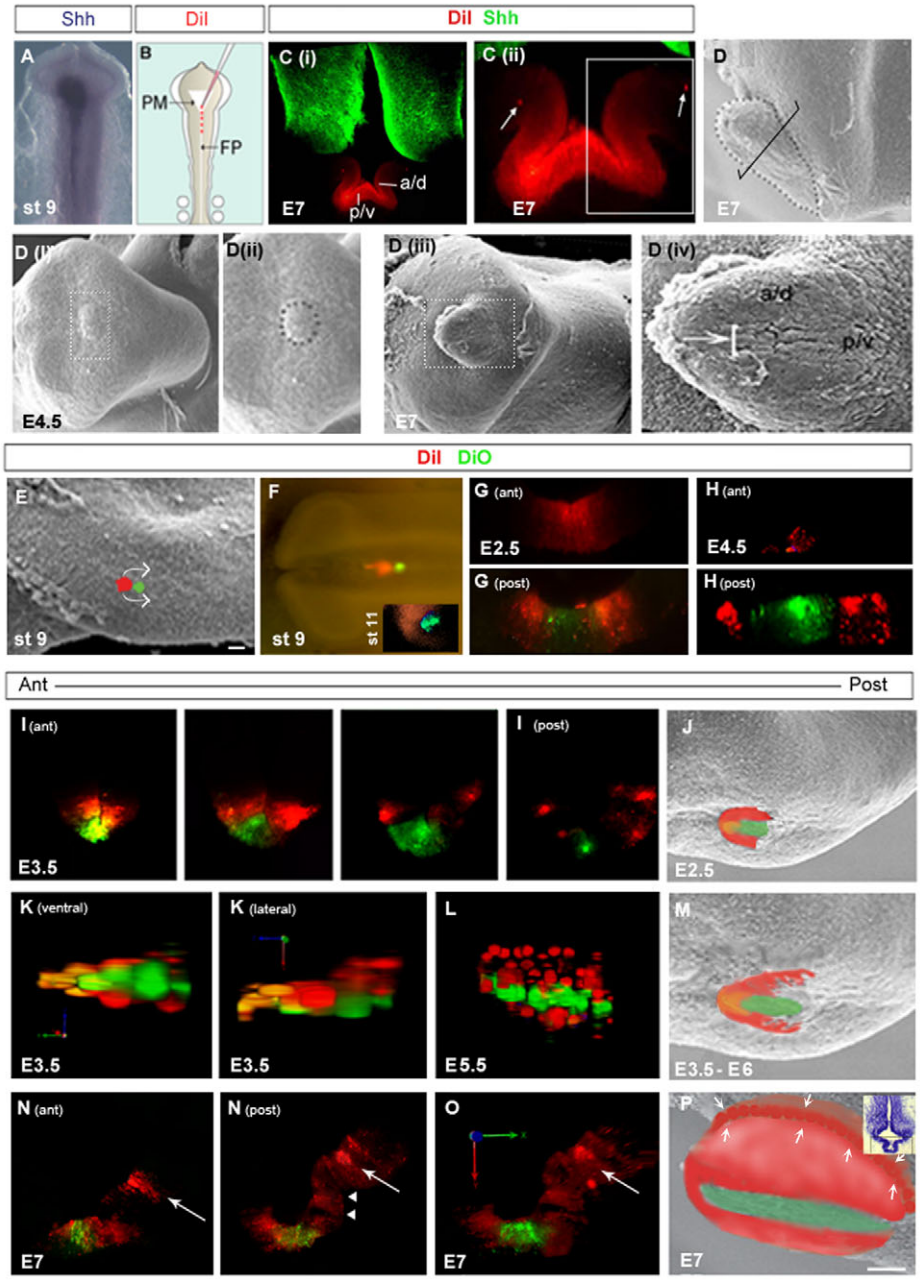

Fig. 1. Dual origin of the infundibulum from the anterior floor plate. (**A**) *Shh* expression in st 9 chick ventral midline floor plate (fp). (**B**) Anterior fp targeted with DiI at st 9. (**C**) A transverse section of an E7 embryo at the level of the infundibulum (indicated in D), after DiI-labelling at st 9. Section is double labelled to detect DiI (red) and *Shh* (green). Low (Ci) and high (Cii) magnification views are shown. DiI labels p/v and a/d regions of the infundibulum and tight cell clusters adjacent to the a/d infundibulum (arrows). The boxed region in Cii indicates the region shown in Fig 1N, Fig. 2R and Fig. 4A,B. (**D**) SEM ventrolateral (D) and ventral (Di-iv) views of infundibulum (outlined in D). The boxed regions in Di and Diii are shown at high magnification in Dii (emerging infundibulum outlined) and Div, respectively. Bracketed arrow in Div points to p/v cells. (**F-I**,**N**) Chick embryos injected with DiI/DiO at st 9 and analysed immediately in wholemounts (F) or in transverse sections through anterior or posterior parts of the infundibulum after successive time periods [F inset, st 11 (8 hours); G, E2.5 (24 hours); I, E3.5 (48 hours); H, E4.5 (72 hours); N, E7 (132 hours)]. I shows a subset of serial sections through an E3.5 infundibulum, used to obtain 3D reconstructions. (**K**,**L**,**O**) Three-dimensional confocal reconstructions at successive time periods viewed ventrally or laterally (K), ventrolaterally (L) or transversely (O). (**E**,**J**,**M**,**P**) SEM views with fate-mapping schematic superimposed. In P, arrows indicate collar cell zone, adjacent to a/d infundibulum and defined through foci of strongly-labelled DiI cells (arrow in N,O). Arrows in E indicate directed movement of prosencephalic neck fp cells; arrowheads in N indicate a/d infundibulum; coloured arrows in K and O indicate viewing axes. FP, floor plate; PM, prechordal mesoderm; a/d, anterior-dorsal infundibulum; p/v, posterior-ventral infundibulum. Scale bars: 100 μm in Ci,Di,Diii; 40 μm in Cii,K,N,O; 50 μm in D,L; 50μm in Div; 30 μm in G; 40 μm in H,I.

Prosencephalic neck descendants were never observed to contribute to the p/v infundibulum. These studies reveal a dual fp origin for the a/d and p/v infundibulum.

FGF and SOX3 expression in the infundibulum and collar

To begin to analyse the molecular profiles of the fp subpopulations and their descendants, we examined the expression of FGF genes previously reported to be expressed in the embryonic ventral hypothalamus. No FGF expression was detected at st 9 (not shown). However, distinct subpopulations of FGF-expressing fp cells were specified at this point: when isolated and cultured to the equivalent of E2.5 (Fig. 2A,B), the more anterior prosencephalic neck (red, Fig. 2A) population expressed *Fgf3* but not *Fgf10* (80% *Fgf3*$^{+}$; 90% *Fgf10*$^{-}$; *n*=20 each; Fig. 2C, top panels), whereas the more posterior population (green, Fig. 2A) was *Fgf3*$^{-}$ *Fgf10*$^{+}$ (100% *Fgf3*$^{-}$; 80% *Fgf10*$^{+}$; *n*=10 each; Fig. 2C, bottom panels).

These characteristics were revealed in vivo at E2.5 and maintained to E7, the latest stage examined. Throughout this period, midline cells expressed *Fgf10*, whereas *Fgf3* was detected in a semi-ellipse of cells increasingly displaced from the midline (Fig. 2D,E,G-I,O,P). Comparison of expression profiles and fate-mapping analyses indicated that fp cells at the prosencephalic neck give rise to *Fgf3*$^{+}$ collar zone cells, whereas posterior fp cells give rise to *Fgf10*$^{+}$ p/v infundibular cells (Fig. 2D-F, E2.5; Fig. 2H,I,I inset, E4.5; Fig. 2O,P,S, E7).

Between E2.5 and E4.5, *Fgf10* expression expanded (compare Fig. 2D with 2H) and by E7 marked the entire infundibulum (Fig. 2M-O). This suggests that, after E2.5, *Fgf10*$^{+}$ cells are derived

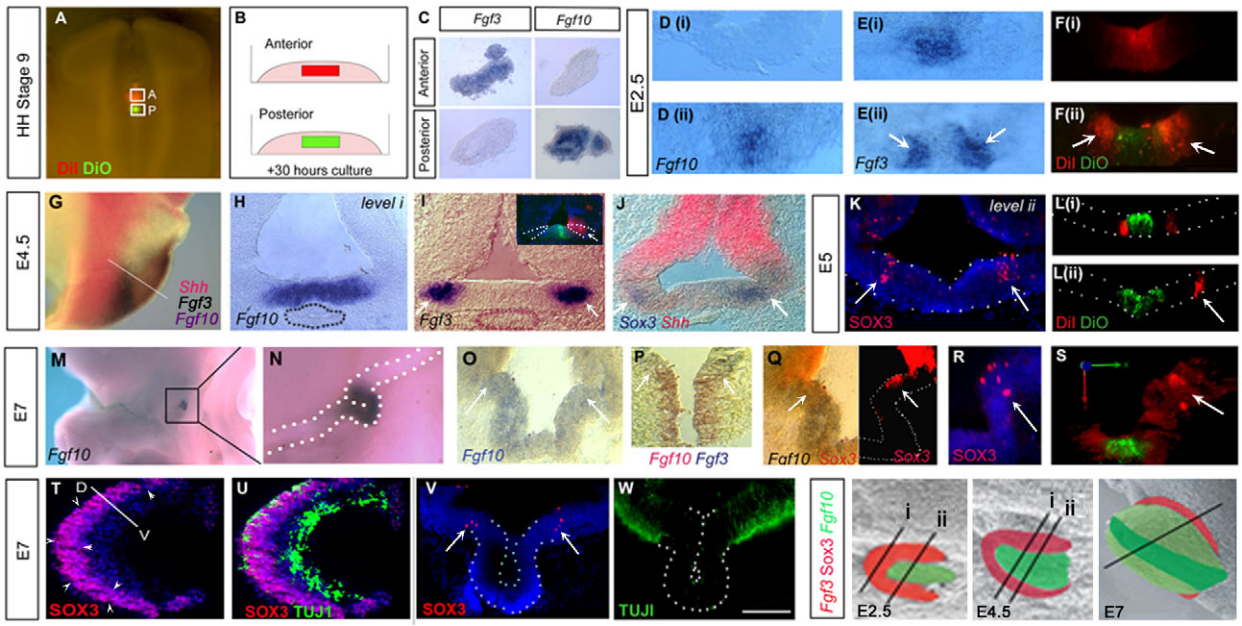

Fig. 2. *Fgf10* and *Fgf3*/SOX3 expression in the forming infundibulum and collar zone. (**A-C**) Anterior fp subpopulations (A and P) used in targeted fate mapping (A) were explanted and cultured in vitro (B) for 30 hours (E2.5 equivalent). The anterior (prosencephalic neck)-derived population expresses *Fgf3* but not *Fgf10*, whereas the adjacent posterior population-derived cells express *Fgf10* but not *Fgf3* (C). (**D-W**) Analysis of infundibulum and collar zone markers in transverse section (D-F,H-L,O-S,V,W), wholemount (G,M,N) or ventral (T,U) views (section levels at specific stages are shown in X, with planes labelled in accordance with panels). (D-F) At E2.5, prosencephalic neck descendants (DiI, red arrows) express *Fgf3* (E,F), whereas posterior fp descendants (DiO, green) express *Fgf10* (D,F). (G) At E4.5, a semi-ellipse of *Fgf3*$^+$ cells lies between the *Fgf10* and *Shh* domains. (H-L) At E4.5-5, posterior fp descendants (green cells in L and in I inset) express *Fgf10*, strongly labelled prosencephalic neck descendants (arrows in L and I inset) are located in *Fgf3*$^+$ *Sox3*/SOX3$^+$ regions (arrows, I,J). (M-O) *Fgf10* marks the infundibulum at E7, its dorsal boundary at the collar zone (arrows, O). (P) Double labelling shows that expression of *Fgf3* and of *Fgf10* abut. Arrow indicates collar zone. (Q) Double labelling shows that expression of *Sox3* and of *Fgf10* abut (single-channel view of *Sox3* shown in the right-hand panel). *Sox3* is expressed in the collar zone (arrows) and ventral hypothalamus. (R) SOX3 is detected only in the collar zone and immediately adjacent cells (arrow). (S) Comparative analysis shows that strongly labelled prosencephalic neck descendants (white arrow) are located in the SOX3$^+$ *Fgf3*$^+$ collar zone and weakly labelled descendants are in adjacent cells in the *Fgf10*$^+$ infundibulum. Coloured arrows in S indicate viewing axes. Comparison of O and S shows that *Fgf10*$^+$ infundibular cells have a dual fp origin. (T) SOX3 in collar zone cells (arrowheads) at E7. (U-W) Co-analysis of SOX3 and TUJ1 in double-labelled wholemount view (U) or serial adjacent sections (V,W). Dotted lines in V and W outline the infundibulum, arrows in V indicate collar zone. TUJ1$^+$ nascent neurons lie dorsal to SOX3$^+$ collar cells. (**X**) Schematics (anterior to left) showing the expression domains of *Fgf3*, SOX3 and *Fgf10* at E2.5, E4.5 and E7. Light green represents *Fgf10*$^+$ prosencephalic neck descendants in the a/d infundibulum. D-V, dorsoventral. Scale bars: 50 μm in C-F; 250 μm in G; 60 μm in H-J; 80 μm in K,L; 200 μm in N; 100 μm in O-W.

from both subsets of fp populations (compare Fig. 2H with 2Li and Fig. 2O with 2S) and that prosencephalic cell descendants contribute to both *Fgf3*$^+$ collar zone cells and prospective/definite *Fgf10*$^+$ a/d infundibular cells. At all times, *Fgf10*$^+$ cells abutted *Fgf3*$^+$ cells (Fig. 2D,E,H,I,P).

We next examined the expression of SOX3, addressing whether it is associated with the infundibulum itself or the collar zone. Prior to E4.5, SOX3 is widely expressed in the prospective collar zone and infundibulum (not shown). However, at ~E4.5-5, SOX3 was extinguished from the majority of the forming infundibulum (Fig. 2J,K) and became restricted to the semi-ellipse of cells in the collar zone and to the dorsal-most cells of the a/d infundibulum (Fig. 2J-L,Q-T). Comparison of *Sox3*/SOX3 expression with that of *Fgf3*, *Shh*, TUJ1 (class III β-tubulin, a marker of early generated neurons in the ventral hypothalamus) and *Fgf10* showed that *Sox3*/SOX3$^+$ cells are in the collar zone (arrows in Fig. 2K,Lii, E5; Fig. 2R,S, E7), bounded dorsally by *Shh* expression at E4.5 (Fig. 2J) and by the TUJ1$^+$ neuronal component of the hypothalamus at E7 (Fig. 2U-W), and that they merge ventrally into *Fgf10*$^+$ infundibular cells (Fig. 2Q). Thus, *Fgf10* expression defines the infundibulum, whereas *Fgf3* and SOX3 largely mark cells in the collar zone and immediately adjacent territories (summarised in Fig. 2X).

Collar cells can contribute to the infundibulum over an extended period

These studies suggest a model in which prosencephalic neck cells give rise to collar zone cells, the descendants of which can populate the a/d infundibulum. However, they do not distinguish this from an alternative model, in which prosencephalic neck cells are a mixed population, some of which differentiate into collar zone cells and others into *Fgf10*$^+$ a/d infundibular cells. To test whether collar zone cells can differentiate to *Fgf10*$^+$ cells, we explanted them at E4 and examined their behaviour in isolation. Immediately after dissection, 100% collar zone explants expressed *Fgf3* and SOX3 but not *Fgf10*, whereas prospective p/v infundibulum explants expressed *Fgf10* but not *Fgf3* or SOX3 (Fig. 3A-C,E-G, not shown; *n*=8 each). However, *Fgf10* expression was robustly upregulated in collar zone explants after 24 hours in culture (Fig. 3D; 9/10 explants were *Fgf10*$^+$).

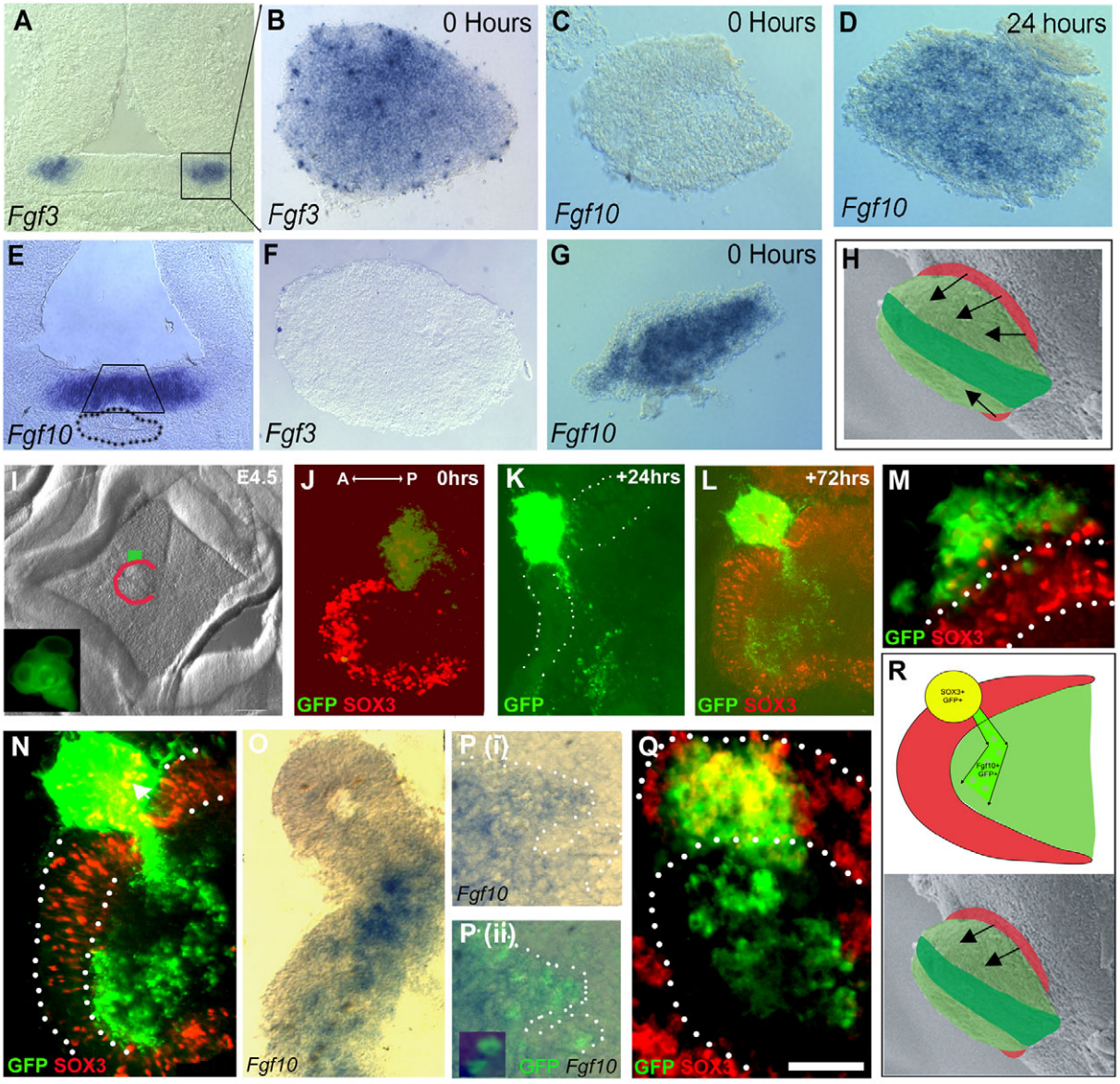

Fig. 3. Collar cell descendants contribute to *Fgf10*$^+$ infundibulum. (**A**) Boxed region indicates dissection of *Fgf3*$^+$ collar zone cells. (**B-D**) Immediately after isolation, collar explants are *Fgf3*$^+$ *Fgf10*$^-$ (B,C) but are *Fgf10*$^+$ after 24 hours in culture (D). (**E-G**) After isolation (E), prospective p/v infundibular cells are *Fgf3*$^-$ (F) and *Fgf10*$^+$ (G). (**H**) Model for infundibular development. p/v infundibulum (dark green) arises from ventral midline floor plate cells that remain in a midline position. *Fgf3*$^+$ SOX3$^+$ collar progenitors (red) can give rise to descendants that populate the a/d infundibulum (light green). Arrows indicate flow of collar descendants to form a/d infundibulum. (**I-L**) Ventral wholemount (I-K) or thick coronal section (L) views of infundibulum/collar region after collar cells from a Roslin-GFP embryo (head shown in inset, I) were transplanted adjacent to endogenous collar cells in wild-type (wt) embryos, shown schematically (I) or analysed after successive time points (J-L). Immediately after transplantation, GFP$^+$ SOX3$^+$ collar cells lie close to the endogenous SOX3$^+$ collar (J). Analysis of a similar graft reveals that GFP$^+$ cells stream medioventrally from the transplant by 24 hours (K, dotted white line indicates the endogenous collar region). The same transplant, analysed at 72 hours (L), reveals more migrating GFP$^+$ cells. Some SOX3$^+$ collar cells remain in situ; migrating cells downregulate SOX3. (**M**) Thick coronal section view after transplantation of E4.5 GFP$^+$ prospective p/v cells. After 72 hours, transplanted cells remain in situ and do not cross the endogenous SOX3$^+$ collar (outlined). (**N-P**) High-magnification view of L (N) and serial adjacent section analysed for *Fgf10* (O). Arrowhead in N points to some SOX3$^+$ collar cells that remain in situ. GFP$^+$ cells migrate into the *Fgf10*$^+$ infundibular region and express *Fgf10* (Pi,Pii). Inset in Pii shows high-magnification view of a GFP$^+$ cell that has upregulated *Fgf10*. (**Q**) Heterochronic transplant from E10 GFP$^+$ to E4.5 wt embryo analysed after 72 hours (thick coronal section). Some GFP$^+$ SOX3$^+$ collar cells remain in situ in the endogenous SOX3$^+$ collar (outlined), whereas other GFP$^+$ cells migrate medioventrally, downregulating SOX3. (**R**) Model showing that collar cell descendants contribute to a/d infundibulum and upregulate *Fgf10*. Top, yellow circle represents the transplanted collar zone. Bottom, see H. A-P, anterior to posterior. Scale bars: 100 μm in A,E; 25 μm in B-D,F,G; 100 μm in H; 300 μm in I; 50 μm in J-L; 60 μm in N,O; 30 μm in P; 50 μm in M,Q.

To directly test whether collar zone descendants can colonise the a/d infundibulum (Fig. 3H), we grafted collar zone cells from GFP-transgenic chicks into explanted wild-type (wt) host hypothalami at E4.5, positioning grafts close to/within endogenous collar cells (Fig. 3I,J). After 24 hours, GFP$^+$ cells appeared to migrate through the endogenous collar and medioventrally into the infundibulum

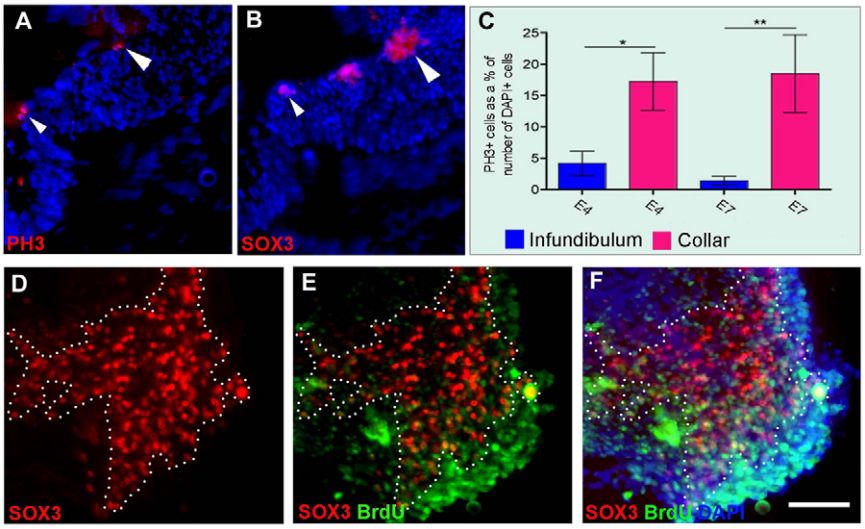

Fig. 4. SOX3$^+$ collar cells proliferate late into embryogenesis. (**A**,**B**) Serial adjacent transverse sections of the infundibulum (showing one half only; see boxed region in Fig. 1Cii). At E7, PH3$^+$ cells (arrowheads, A) are almost entirely restricted to SOX3$^+$ collar cells (arrowheads, B) and/or immediately adjacent cells in a/d infundibulum. (**C**) Comparative statistical analyses demonstrate a significantly higher proportion of mitotic cells in the collar and immediately adjacent region than in the distal infundibular regions at E4 and E7. *, P=0.0305; **, P=0.0076. Error bars indicate s.e. (**D-F**) Isolated explants of E6 collar region/infundibulum, cultured and pulsed for 1 hour with BrdU and analysed with anti-SOX3, anti-BrdU and DAPI. SOX3$^+$ cells (outlined) are detected (D). The overlay (E,F) reveals SOX3$^+$ BrdU$^-$ (red), SOX3$^+$ BrdU$^+$ (yellow), SOX3$^-$ BrdU$^+$ (green) and SOX3$^-$ BrdU$^-$ (blue) cells. Scale bars: 80 μm in A,B; 70 μm in D-F.

(Fig. 3K). Explants were fixed after 72 hours to examine the expression of collar and infundibular markers and confirm this behaviour. These analyses revealed that some GFP$^+$ SOX3$^+$ collar zone cells had remained in situ, with cells closest to the endogenous collar continuing to express SOX3 (Fig. 3L,N, arrowhead). Additionally, GFP$^+$ cells had migrated through the endogenous collar zone into the *Fgf10*$^+$ infundibulum (Fig. 3L,N,O). The stream of GFP$^+$ cells had increased in number relative to 24 hours previously (compare Fig. 3K with 3L). Migrating cells emanating from the graft did not express SOX3 but upregulated *Fgf10* (Fig. 3O,P) and did not appear to re-enter the endogenous SOX3$^+$ collar zone, suggesting that cells differentiating from collar zone cells remain spatially separate from them. In addition to the robust flow of cells into the infundibulum, an occasional GFP$^+$ cell with neuronal morphology extended dorsally from the graft (not shown). These experiments show that collar zone cells can differentiate, giving rise to cells that colonise the infundibulum and that upregulate *Fgf10*.

The maintained expression of SOX3 and *Fgf3* in the collar zone suggests that collar zone cells are retained late into embryogenesis; we therefore asked whether they can contribute progeny to the infundibulum over an extended period. Heterochronic (E10 GFP$^+$ to E4.5 wt) grafting experiments showed that E10 collar cells display identical behaviour to those at E4.5: some remained in situ and maintained expression of SOX3, whereas some migrated into the infundibulum (Fig. 3Q). Thus, collar cells retain the ability to populate the infundibulum into late embryogenesis.

Finally, we addressed whether the directed migratory behaviour is restricted to collar zone cells or whether *Fgf10*$^+$ p/v infundibular cells behave similarly. GFP$^+$ *Fgf10*$^+$ p/v infundibular cells were subdissected (Fig. 3E), similarly grafted (Fig. 3I) and examined after 3 days. Such grafts did not invade the SOX3$^+$ collar zone nor did they migrate beyond it into the infundibulum (Fig. 3M). Together, these experiments suggest that the extended growth of the infundibulum relies on a protracted inflow of collar zone-derived descendants (schematised in Fig. 3R).

Differential cell proliferation in infundibular formation

The idea that cells from the collar zone contribute to the a/d infundibulum over a period of days, together with our observations that relatively weak Di labelling is detected in a/d as compared with p/v infundibular cells, suggest that collar zone cells, or their progeny, might undergo extensive proliferation. To test this directly, we analysed proliferation in the collar region and infundibulum (Fig. 4A-C). Analysis of phosphohistone H3 (PH3), a marker of G2/M-phase cells, confirmed that some SOX3$^+$ collar cells and their immediate neighbours remain in the cell cycle in vivo and proliferate late into embryogenesis. At E4, significantly higher numbers of PH3$^+$ cells were detected in the collar cell region and the immediately adjacent a/d infundibulum than in the p/v infundibulum (Fig. 4C, n=5 embryos, P<0.05). By E7, PH3$^+$ cells were confined almost exclusively to SOX3$^+$ collar cells and/or their immediate neighbours (Fig. 4A-C; 153 PH3$^+$ cells examined in eight embryos, P<0.05). Thus, SOX3$^+$ collar cells and their close neighbours proliferate more extensively than p/v infundibular cells.

To provide further evidence that SOX3$^+$ collar cells are a proliferating population, we performed BrdU labelling experiments in vitro. Explants composed of collar and infundibular cells were dissected from E6-7 embryos, cultured with BrdU and then double labelled to detect the relative positions of BrdU$^+$ and SOX3$^+$ cells. BrdU$^+$ cells were always closely associated with SOX3$^+$ collar cells. A consistent pattern of proliferation was detected: central-most SOX3$^+$ BrdU$^-$ cells (55% of SOX3$^+$ cells) surrounded by SOX3$^+$ BrdU$^+$ cells (45% of SOX3$^+$ cells), then SOX3$^-$ BrdU$^+$ cells (83% of BrdU$^+$ cells) and finally SOX3$^-$ BrdU$^-$ cells (Fig. 4D-F; n=5).

Collar cells proliferate extensively in vitro and can differentiate to multiple fates

To further test the proliferative capacity of collar versus p/v infundibular cells, we analysed the ability of each to form neurospheres (Deleyrolle and Reynolds, 2009) and, for comparison, anterior spinal cord fp cells and tail bud. Primary neurospheres could be derived from collar cells and tail bud but not from the p/v infundibulum or anterior spinal cord fp (Fig. 5A; not shown). Lineage-tracing studies confirmed the collar cell origin of neurosphere-producing cells (Fig. 5B). Primary collar cell-derived neurospheres expressed SOX3, *Fgf3* and *Pea3*, suggesting that they retained their original undifferentiated collar cell character (Fig. 5C-E, Fig. 6C). Collar cell neurospheres, moreover, expressed SOX2, *Notch1* and Musashi1 (Fig. 5F-I), which are markers associated with neural stem/progenitor cells, and found in the ventricular zone of the hypothalamus, including the collar zone (not

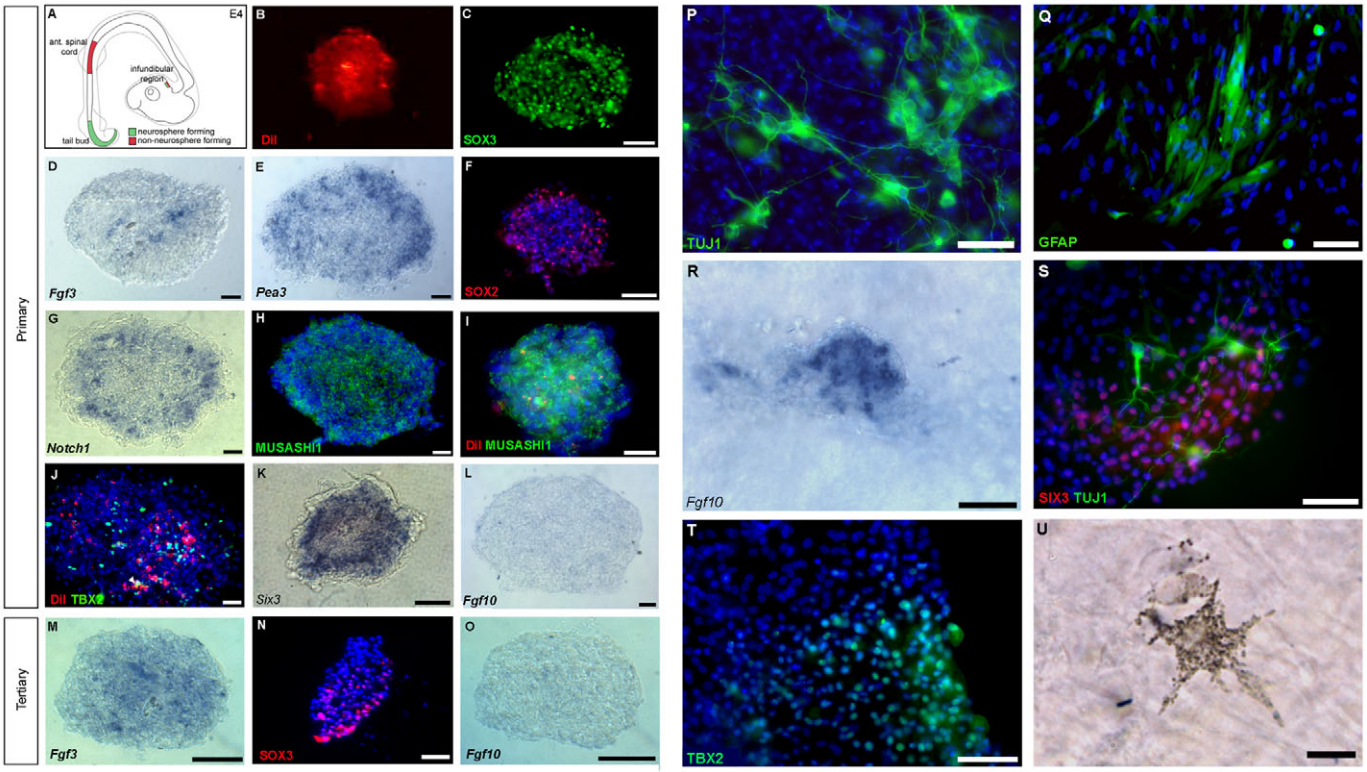

Fig. 5. Neurosphere analysis. (**A**) Schematic of neurosphere-forming (green) and non-forming (red) regions of the chick neuraxis at E4. (**B-L**) Marker expression in collar primary neurospheres. (B,I,J) Collar zone neurospheres generated from DiI-labelled prosencephalic neck fp cells. DiI expression is retained and is adjacent to/overlaps with Musashi1$^+$ TBX2$^+$ cells (white arrowhead in J indicates DiI$^+$ TBX2$^+$ cells). (**M-O**) Tertiary neurospheres maintain expression of *Fgf3* and SOX3 but do not express *Fgf10*. (**P-R**) Single primary neurospheres cultured in reduced EGF for 7-10 days can differentiate into TUJ1$^+$ neurons, GFAP$^+$ cells and *Fgf10*$^+$ cell clusters. (**S**,**T**) Expression of the anterior marker SIX3 (red) is maintained in clusters of cells that form TUJ1$^+$ neurons. Note that SIX3 expression is excluded from TUJ1$^+$ neurons. TBX2 expression is maintained in a few cells. (**U**) Differentiated neurospheres can give rise to pigmented cells. Scale bars: 50 μm in B-T; 20 μm in U.

shown). In addition, they expressed TBX2 and *Six3* (Fig. 5J,K), which are markers of the ventral diencephalon and hypothalamus, respectively (Kobayashi et al., 2002; Ohyama et al., 2005; Manning et al., 2006; Pontecorvi et al., 2008), suggesting that they maintain hypothalamic regional identity. However, no expression of *Fgf10* was detected in undifferentiated primary spheres (Fig. 5L).

To address whether collar neurospheres can self-renew, we passaged them repeatedly. In the presence of FGF2 and EGF, neurospheres could be re-derived for up to five passages (the maximum tested). Analyses of re-derived undifferentiated neurospheres revealed that, similar to primary neurospheres, they retain expression of *Fgf3*, *Pea3* and SOX3, but do not express *Fgf10* (Fig. 5M-O; not shown).

To ascertain that, as in vivo, neurospheres can differentiate to an infundibular fate, growth factors were reduced. Single neurospheres differentiated into multiple fates (Fig. 5P-R), including *Fgf10*$^+$ cells (Fig. 5R). These formed cup-like structures, reminiscent of the architecture of the forming infundibulum. Additional markers suggested that differentiated cells maintained hypothalamic character. TUJ1$^+$ neurons differentiated within SIX3$^+$ areas (Fig. 5S). TBX2 expression was likewise retained in a small number of cells, in keeping with its reduced expression in vivo (Fig. 5T) (Pontecorvi et al., 2008). Finally, pigmented cells sporadically differentiated throughout the cultures, suggestive of the presence of melanin-concentrating hormone cells that are found in vivo in the ventrolateral hypothalamus (Fig. 5U) (Coll et al., 2004). Together, these analyses reveal that collar cells can proliferate extensively ex vivo and can differentiate to multiple fates, including hypothalamic infundibular cells.

FGF signalling is required to maintain SOX3$^+$ proliferating progenitors

Given that elsewhere in the CNS, SOX expression is dependent on FGF signalling (Wilson et al., 2000; Streit et al., 2000; Takemoto et al., 2005; Stavridis et al., 2007; Rogers et al., 2008; Ishii et al., 2009; Tucker et al., 2010), we hypothesized that FGF signalling might also regulate collar zone cell proliferation by maintaining SOX3$^+$ progenitors.

Analysis of FGF signalling pathway components revealed that FGFR1, but not FGFR2 or FGFR3, is expressed in the developing collar region and infundibulum over the period E4-7 (Fig. 6A,B; not shown). Additionally, the transcriptional targets *Pea3* and *Erm*, and dual phosphorylated MAPK (p42p44pMAPK, termed pMAPK), a readout of ERK1/2 activation, were expressed throughout this time (Fig. 6C-E; not shown). *Pea3* and pMAPK were notably absent from p/v infundibular cells and were restricted to the collar region and immediate a/d infundibulum (Fig. 6C-E).

To examine whether FGF signalling can maintain SOX3$^+$ cells, we cultured explants composed of collar region and immediately adjacent cells from E4-5 embryos alone or with the FGF inhibitor SU5402. SU5402 treatment over 24 hours was effective in reducing FGF signalling, leading to a 65% decrease in the number of

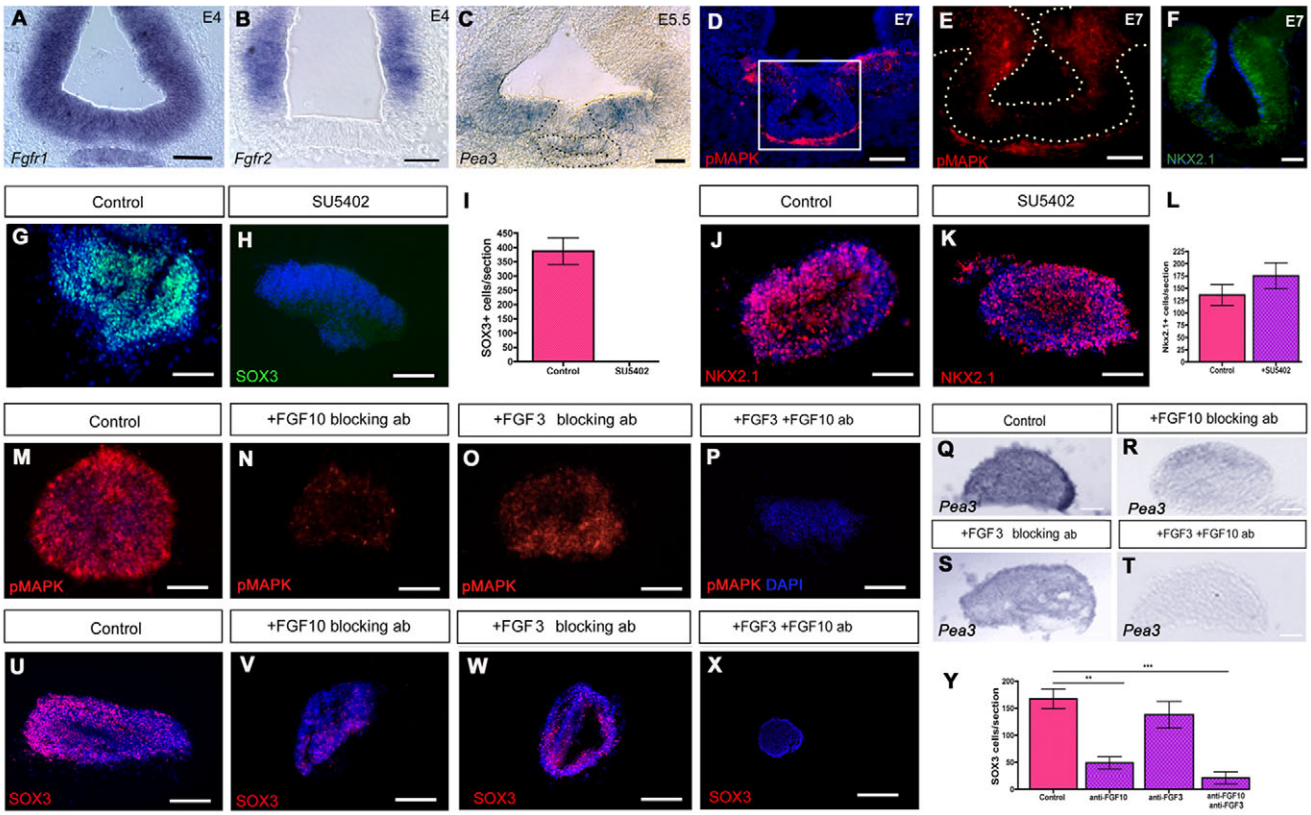

Fig. 6. FGF signalling maintains SOX3$^+$ progenitors. (**A-E**) Transverse sections showing expression of FGF signalling components. The forming p/v infundibulum and Rathke's pouch are outlined (C). The boxed region in D is shown at higher magnification in E. (**F**) Expression of NKX2.1 in the infundibulum. (**G-T**) Explants of collar/infundibulum, dissected at E4, cultured alone or with FGF inhibitors. (G-L) SU5402 significantly affects the number of SOX3$^+$ cells but not the number of NKX2.1$^+$ cells. (M-P) Many pMAPK$^+$ cells are seen in control explants (514±39.7), fewer with FGF3-blocking antibody (321±11.45), and significant reductions are detected in the presence of FGF10-blocking antibody (134±5.8) or both blocking antibodies (15±14.6). (Q-T) *Pea3* expression declines after exposure to FGF3 antibody, still further after exposure to FGF10 antibody, and is not detected after exposure to both antibodies. (**U-Y**) SOX3 cell number declines after exposure to FGF3 antibody, still further after exposure to FGF10 antibody, and few SOX3$^+$ cells are detected after exposure to both antibodies. Error bars indicate s.e. *, P=0.0006; **, P=0.0001. Scale bars: 50 μm in A,B,E-K,M-X; 60 μm in C; 100 μm in D; 40 μm in Q-T.

pMAPK-expressing cells and similarly reduced the extent of *Pea3* expression (n=40; not shown). Treatment over 24 hours resulted in a complete loss of SOX3$^+$ cells, without a similar immediate decrease in NKX2.1$^+$ infundibular progenitors (Fig. 6G-L). This indicates that FGF signalling may selectively maintain SOX3$^+$ cells, rather than exerting a generalised effect on all progenitor cells.

Although SU5402 is widely used as an inhibitor of FGF signalling, it does not distinguish whether it is FGF10, FGF3 or both ligands that contribute to SOX3$^+$ cell maintenance. We therefore analysed the effects of FGF10- or FGF3-blocking antibodies by culturing explants alone or in the presence of either or both antibodies. In the absence of FGF10 signalling there was a significant decrease in *Pea3* expression and in the number of pMAPK$^+$ and SOX3$^+$ cells (P<0.0001 and P<0.0006, respectively). By contrast, blockade of FGF3 signalling resulted in a less severe decrease in *Pea3* expression and in pMAPK$^+$ and SOX3$^+$ cell number (P<0.0035 and P<0.046, respectively). However, exposure of explants to both blocking antibodies together resulted in a substantial reduction or the complete loss of *Pea3* expression and in a highly significant decrease in pMAPK$^+$ and SOX3$^+$ cell number (P<0.0001 for both; n=8-10 explants each; Fig. 6M-Y). This suggests that SOX3$^+$ cells might require both FGF3 from the collar region and FGF10 from the forming infundibulum.

Studies in mouse have shown that expression of *Fgf3* in the ventral forebrain is governed, at least in part, by SHH (Powles et al., 2004), raising the possibility that collar cells are SHH dependent. To examine this in chick, we dissected st 9 prospective collar cells (Fig. 7A) and cultured them alone or with cyclopamine, an inhibitor of SHH signalling. Cyclopamine treatment reduced the expression of SOX3 and *Fgf3* after a short culture period and of *Fgf10* after protracted culture (Fig. 7B,C). Thus, as with many other hypothalamic progenitors (Ohyama et al., 2008), collar cell induction appears to depend on early SHH signalling. We next used this assay to establish whether FGF ligands can expand collar cells, rescuing their numbers after a reduction in SHH signalling. Exposure of prospective collar cells to a combination of cyclopamine and FGF10 resulted in a partial rescue of SOX3-expressing cells and *Fgf3* expression. Moreover, after an extended period, *Fgf10* expression was detected in cells protruding from the main body of the explant (Fig. 7D,E). These experiments support the idea that FGF10 expands collar cells and suggests that FGFs might govern collar cell proliferation.

To test this latter contention directly, we asked whether the loss of SOX3$^+$ cells following a reduction in FGF signalling is accompanied by a decrease in progenitor cell proliferation. Treatment of E4.5 collar region explants with SU5402 not only

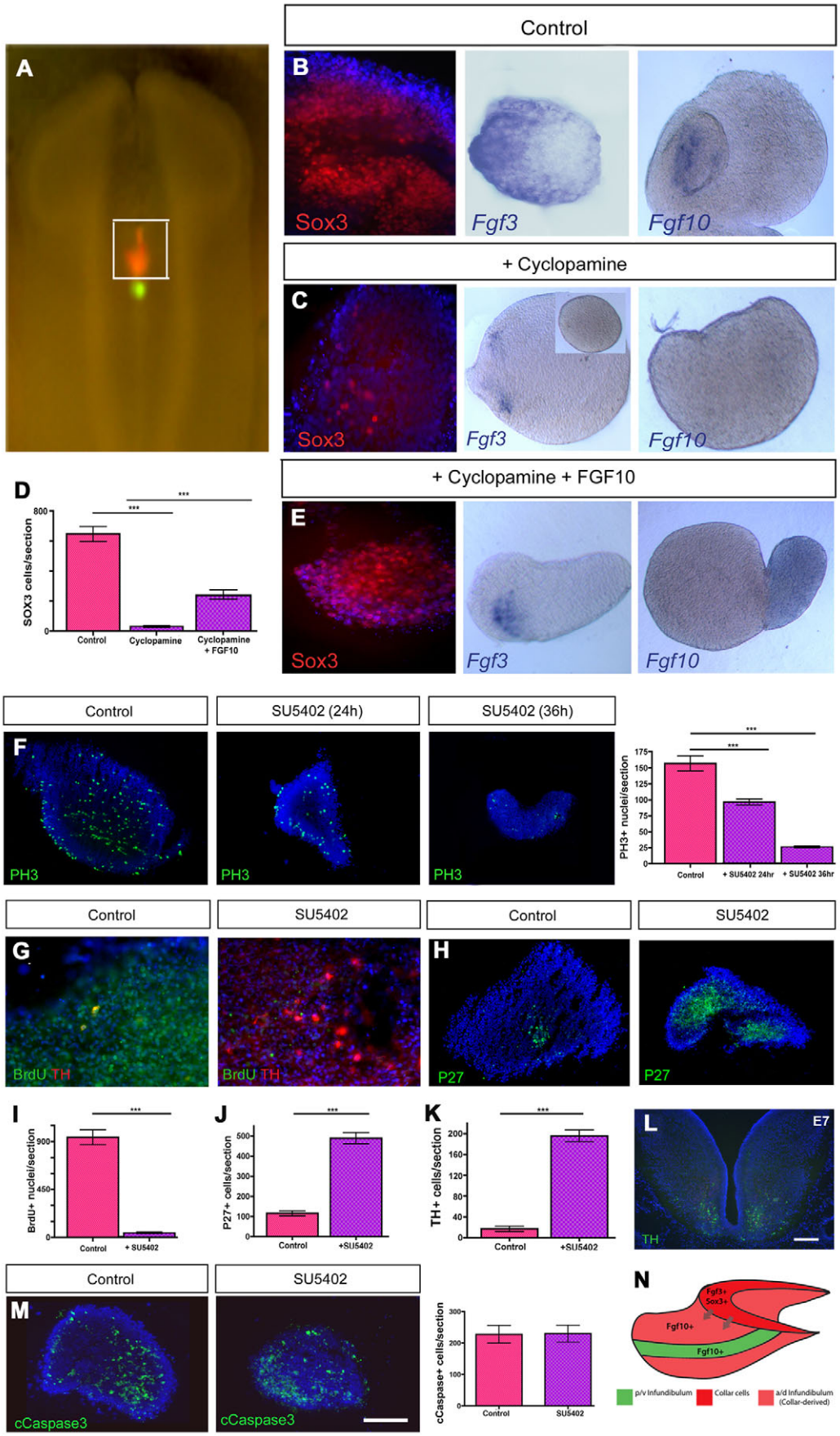

Fig. 7. FGF signalling promotes proliferating collar progenitors. (**A-E**) St 9 prosencephalic neck cells explanted and cultured in vitro. In control explants (B), collar cells expressing SOX3 and *Fgf3* are detected after 48 hours, and an *Fgf10*$^{+}$ protrusion is detected after 7 days. With cyclopamine (C), SOX3 and *Fgf3* expression is markedly reduced or abolished (inset) and no expression of *Fgf10* is detected. Expression of SOX3, *Fgf3* and *Fgf10* is partially rescued in explants exposed to both cyclopamine and FGF10 (E). (D) Quantitation of SOX3 analyses. Control, 652±40 SOX3^{+} cells; with cyclopamine, 18±2 SOX3^{+} cells; with cyclopamine and FGF10, 220±25 SOX3^{+} cells. Error bars indicate s.e.; ***, P<0.0001. (**F-K**) Explants of E4 collar/adjacent a/d infundibulum, cultured alone or with SU5402. (F) Treatment with SU5402 reduces PH3-expressing cells by 24 hours and eliminates them by 36 hours. (G,I) In explants exposed to BrdU for 4 days, little or no incorporation of BrdU is detected after SU5402 treatment. Conversely, TH and P27 expression is increased in the absence of FGF signalling (G,H,J,K). (**L**) Expression of TH in the arcuate nucleus. (**M**) No change is detected in cCaspase 3 activity after SU5402 treatment. Error bars indicate s.e. ***, P<0.0001 in F,I,K and P<0.005 in J. (**N**) Schematic of infundibulum. p/v infundibular cells (green) are induced by Shh/Nodal, and specified by BMP/Wnt signalling; collar zone cells are induced by Shh, maintained by FGF signalling; collar-derived a/d infundibular cells (light red) are specified by an unknown signal. Scale bars: 40 μm in B-E,L; 60 μm in F,H,M; 30 μm in G.

eliminated SOX3 expression (Fig. 6G,H) but led to a significant decrease in cycling cells (Fig. 7F). A 50% reduction in $PH3^+$ cells was observed after 24 hours and a 90% reduction after 36 hours (n=5, P<0.0001). Similarly, when BrdU was administered to control or SU5402-treated explants, significantly fewer $BrdU^+$ cells were found following a reduction in FGF signalling (n=6, P<0.0001; Fig. 7G,I). Reduced proliferation was accompanied by enhanced differentiation: we detected a highly significant increase in p27, a marker of post-mitotic cells (n=7, P<0.001; Fig. 7H,J), and in tyrosine hydroxylase $(TH)^+$ dopaminergic neurons that differentiate in the ventral hypothalamic arcuate nucleus (Fig. 7G,K,L), after reduction of FGF signalling. Exposure to SU5402 did not appear to promote an increase in cell death: no difference was detected in the rate of apoptosis, as measured through activated cleaved (c) Caspase 3 activity (n=5, P=0.96; Fig. 7M), in control versus SU5402-exposed explants. Together, these results suggest that decreased FGF signalling leads to a reduction in collar cell proliferation.

DISCUSSION

The infundibulum plays a pivotal role in vertebrates, linking the nervous and endocrine systems, and its proper development is crucial to homeostasis. Previous studies have suggested that the infundibulum derives from the ventral midline of the hypothalamus and that its development is triggered through early signalling events between the hypothalamic midline and Rathke's pouch (Pelletier, 1991; Dasen et al., 2001; Hermesz et al., 2003; Rizzoti et al., 2004). Here, we demonstrate that two anterior fp subsets that are initially induced by Nodal and SHH signalling (for a review, see Placzek and Briscoe, 2005) fashion the infundibulum, governing its protracted sculpted evagination. The p/v infundibular cells derive from a set of anterior fp cells that are specified through BMP/Wnt signals to express *Fgf10* (Fig. 7N) (Kapsimali et al., 2004; Manning et al., 2006) and that remain in a ventral midline location. By contrast, a/d infundibular cells derive from a second subset of anterior fp cells that migrate laterally and posteriorly to form a collar of cells around the forming p/v infundibulum. $SOX3^+$ collar cells are a proliferative neural progenitor population that, although initially induced by SHH, is dependent on FGF signalling. In addition to proliferation, collar cells can differentiate to multiple fates, including $Fgf10^+$ cells that populate the a/d infundibulum (Fig. 7N). Proliferating collar cells are retained at the junction of the infundibulum and hypothalamus at least until late into embryogenesis. These findings have implications for the development, function and maintenance of the infundibulum.

Dual origin of the infundibulum

Many lines of evidence in our study support a dual fp origin for $Fgf10^+$ infundibular cells. Our fate-mapping studies reveal that one set of anterior fp cells remains at the midline, giving rise to cells that populate the p/v infundibulum, whereas a second adjacent set ('prosencephalic neck' cells) gives rise to a collar of cells, from which the a/d infundibulum forms. The two fp populations display markedly different behaviours. Forming p/v infundibular cells do not proliferate extensively, as evidenced by the retention of strong lineage label expression and a lack of active mitosis, and their descendants remain at the ventral midline: little or no cell mixing is observed in double fate-mapping analyses and grafting experiments show that prospective p/v infundibular cells do not contribute to the a/d infundibulum. The final fate of p/v infundibular cells in unclear, but a likely possibility is that they give rise to the posterior pituitary/neurohypophysis, which is the region in the later embryo to which magnocellular hypothalamic axons project.

By contrast, cells of the a/d infundibulum form from a population of cells that undergo extensive migration and proliferation. The behaviour of isolated explants (Fig. 7A,B) suggests that prosencephalic neck cells intrinsically migrate posteriorly/laterally to form collar zone cells. Some cells in the collar zone appear to proliferate little or slowly, as judged by label retention; however, in general, extensive proliferation occurs in the collar zone. Our observations raise the possibility that the collar zone has aspects of a stem cell-like niche, in which slowly dividing cells can give rise to rapidly proliferating progenitors, some of which differentiate to a/d infundibular fates. Neurosphere analyses, explant culture and grafting studies support this interpretation, showing that collar zone descendants can differentiate into multiple fates, including $Fgf10^+$ cells that colonise the a/d infundibulum.

Although we cannot exclude the possibility that there is an additional source of cells that contributes to the a/d infundibulum, our data strongly suggest a model in which cells of the a/d infundibulum derive from collar cells, which in turn originate from prosencephalic neck fp. Our grafting studies show, moreover, that the collar zone is able to contribute cells to the $Fgf10^+$ infundibulum over an extensive period of time, at least until E10. This observation suggests that, in the late embryonic period, collar zone cells that now lie at the interface of the infundibulum and hypothalamus can continue to shape and/or maintain the infundibulum.

FGF signalling governs proliferating $SOX3^+$ collar progenitors

Emerging studies from a number of vertebrates suggest that FGF signalling plays a pivotal role in development of the neuroendocrine hypothalamus and the pituitary gland, and raise the notion that FGFs might govern the development of the infundibulum itself, potentially via effects on proliferation (for a review, see Tsai et al., 2011). Our studies suggest a mechanism for FGF function in infundibular growth, showing that FGF signalling is necessary for the proliferation of collar cells.

Our studies reveal, though, that the collar zone is not a homogeneous population, and we cannot unequivocally establish which cells respond directly to FGF signalling, nor which collar zone cell type gives rise to a/d infundibular cells. However, in the mouse, SOX3 has been shown to play a crucial role in infundibular development (Rizzoti et al., 2004) and our observations support the view that $SOX3^+$ cells play an intimate role in a/d infundibular formation in chick: in cyclopamine-treated explants, the reduction of SOX3 is accompanied by a reduction in $Fgf10^+$ cells; conversely, the rescue of SOX3 is accompanied by the rescue of $Fgf10^+$ cells.

Our studies demonstrate, furthermore, that $SOX3^+$ collar cells are proliferative progenitors. In vivo, $SOX3^+$ cells are maintained late into embryogenesis and are mitotically active, as evidenced by detection of PH3. In vitro, $SOX3^+$ cells can proliferate, as judged by uptake of BrdU, but are not depleted, their numbers remaining relatively constant between E5 and E7. Notably, only a subset of $SOX3^+$ cells appears to undergo rapid division. This, together with the observation from the fate-mapping studies that $SOX3^+$ cells span label-retaining collar zone regions and immediately adjacent label-diluted regions in the dorsal-most a/d infundibulum, suggest differential proliferation in subsets of $SOX3^+$ cells. The existence of distinct subsets of $SOX3^+$ cells could account for the lack of any apparent change in SOX3 expression in the *Lhx2*-null mouse, in

which inappropriately high levels of proliferation are detected in the ventral diencephalic floor, with concomitant failure of infundibular evagination (Zhao et al., 2010).

How SOX3 exerts its actions and the nature of SOX3$^+$ cells remain unclear. Several members of the SOX family are expressed in neural stem and progenitor cells, where they are thought to play crucial roles in cell proliferation and in the maintenance of the neural stem and progenitor state (Pevny and Placzek, 2005; Scott et al., 2010). SOX family members operate in a context-dependent manner, interacting with partner proteins, including other SOX proteins, to effect their actions. It seems likely that additional SOX proteins might play a role in the collar zone, interacting with SOX3$^+$ cell subsets.

Proliferating SOX3$^+$ collar cells are dependent on FGF signalling. A reduction in FGF signalling results in the loss of SOX3$^+$ cells and in the gradual depletion of proliferative progenitors. The decrease in proliferation does not appear to be accompanied by changes in apoptosis, but instead by an increase in differentiated cells. Conversely, SOX3$^+$ cells can be rescued by FGF10 after cyclopamine treatment. Although we cannot prove that FGF operates directly on SOX3$^+$ cells, FGF signalling is not simply a permissive proliferative signal for all progenitor cells: NKX2.1$^+$ infundibular progenitors are not acutely affected by reduced levels of FGF. Our studies are therefore consistent with a model in which SOX3$^+$ cells can either proliferate or are capable of giving rise to differentiated progeny, including descendants that contribute to the a/d infundibulum over an extended period. As yet, we do not understand how collar cells normally differentiate to infundibular cells, but the downregulation of FGF signalling components, including *Pea3* and pMAPK, in differentiating collar cells that enter the a/d infundibulum suggests a loss of competence to FGF signalling.

One likely early source of FGFs for the maintenance of collar cells is the p/v infundibular cell population. This suggests a novel role for ventral midline FGF10$^+$ cells: signalling to adjacent floor plate-like cells to sustain them in a proliferative state. The finding that FGF signalling from ventral midline cells may sustain proliferative properties in adjacent lateral fp cells might be relevant in other regions of the neuraxis. SoxB1 gene expression is maintained in lateral fp cells that show sustained proliferation (Pevny et al., 1998), whereas expression of FGFs, namely *Fgf3* and the isoform *altFgf2*, has been noted in ventral midline fp cells in the posterior neuraxis (Shi et al., 2009; Borja et al., 1996).

Our neurosphere analyses and grafting experiments show that collar cells can give rise not just to infundibular cells, but also to neuronal cells. This suggests that anterior fp cells that lie at the prosencephalic neck at st 9 have neuronal potential, a property previously ascribed only to midbrain ventral midline fp cells (Hynes et al., 1995; Andersson et al., 2006; Ono et al., 2007), and that, in addition to their role in infundibular formation, collar cells might contribute to, and shape, neuronal components of the hypothalamus.

In summary, our data suggest the presence of a spatially restricted progenitor zone that forms around the anterior end of ventral midline cells of the neural tube. We propose that this zone shapes both the infundibulum and, potentially, the overlying hypothalamus, and that it can contribute cells to the infundibulum over an extended period. Intriguingly, other studies have shown that there is a second proliferative zone at the most caudal region of the forming neural tube, in which FGF signalling maintains cells in a proliferative, undifferentiated state (Diez del Corral et al., 2003; Delfino-Machin et al., 2005; McGrew et al., 2008). The two ends of the neural tube therefore share the ability to promote FGF signalling and establish proliferative zones.

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Competing interests statement
The authors declare no competing financial interests.

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